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Intravital Imaging of the Kidney Using Multiparameter Multiphoton Microscopy

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Abstract
Intravital optical microscopy provides a powerful means of studying the cell biology in the most physiologically relevant setting. The ability of multiphoton microscopy to collect optical sections deep into biological tissues has opened up the field of intravital microscopy to high-resolution studies of multiple organs. Presented here are examples of how two-photon microscopy can be applied to intravital studies of kidney physiology and the study of disease processes. These include studies of cell vitality and apoptosis, fluid transport, receptor-mediated endocytosis, blood flow, and leukocyte trafficking. Efficient two-photon excitation of multiple fluorophores permits comparison of multiple probes and simultaneous characterization of multiple parameters. Two-photon microscopy can now provide a level of investigation previously unattainable in intravital microscopy, enabling kinetic analyses and physiological studies of the organs of living animals with subcellular resolution. Therefore, application of this technology will provide direct visualization of organ-specific and cell-specific responses to an array of stimuli and therapeutic approaches, enhancing our understanding and treatment of disease processes.

Introduction

The kidney is an extremely complex heterogeneous organ consisting of well-defined vascular and epithelial components functioning in the highly coordinated fashion to allow for the fine regulation of a myriad of interdependent processes. Over the years researchers have developed novel and imaginative experimental approaches that allow for understanding and integrating the unique structure-function relationships that occur within the kidney. Investigators have developed model systems to enable enhanced manipulation and isolation of specific variables of interest. However, these models are not without their shortcomings and, although absolutely essential, lack the organ-specific complexity necessary for the final understanding of a disease process. Models also severely limit our ability to test therapeutic approaches to disease states.

A way of circumventing these shortcomings is to study cells within living animals. This approach has been signif-

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icantly advanced by the development of multiphoton microscopy which provides the means of quantitatively analyzing cellular physiology and pathophysiology in the context of an intact functioning organ. The studies described demonstrate the enormous potential of multiphoton imaging of the kidney.

Multiphoton Microscopy and Intravital Microscopy

Light microscopy has become essential to an integrated approach to modern biomedical research. With the development of new technologies, including a wide variety of fluorescent probes, light microscopy enhanced our understanding of the subcellular organization of particular molecules by allowing biochemical and molecular experiments to be conducted within individual cells. The recent confluence of technical developments in fluorescent probes, optics, laser technology, digital imaging, and computer technology provides biomedical researchers with unprecedented opportunities to explore biology using light microscopy.

Where once optical microscopy was limited to analysis of thin cells and tissue sections, the development of confocal microscopy and digital deconvolution microscopy made three-dimensional microscopy possible. However, the potential to image deep into biological tissues was not realized until the development of multiphoton microscopy [1, 2]. The increased penetration of multiphoton microscopy results from the unique photophysics of multiphoton fluorescence excitation, in which fluorescence is stimulated by the simultaneous absorption of two low-energy photons by a fluorophore. The probability of this event is such that the requisite flux of photons occurs only at the focal point. Since light scatter decreases at long wavelengths, the use of near-infrared light in multiphoton microscopy increases the depth of illumination of a scattering biological sample. More significantly, since fluorescence is excited only at the focal point, multiphoton microscopes require no confocal pinhole to collect high-resolution optical sections and consequently collect scattered fluorescence more efficiently.

Multiphoton microscopy has been previously used in intravital studies of morphology and physiology of the brain [3–8], metabolism of the skin [9], development and vascularization of tumors [10], and embryonic development [11]. Recently, the extended optical reach provided by multiphoton microscopy was applied to imaging of the kidneys of living rats [12]. Previous intravital studies of the kidney utilized conventional fluorescence, reflected-light, and transillumination microscopy to characterize organic anion transport [13, 14], tubular obstruction [15], microcirculation, and tubular fluid flow [16–19]. Multiphoton microscopy is capable of providing subcellular resolution deep into functioning, intact kidneys, providing an unprecedented window into cell biology, biochemistry, and physiology of the kidney in the context of the intact functioning organ [12].

Intravital Microscopy of the Intact Kidney Using Multiphoton Microscopy

Multiple fluorophores can be excited by a single excitation wavelength of 800 nm. The ability to image multiple colors of fluorescence was key to characterizing multiple parameters in the imaged kidneys, and also to identifying endogenous autofluorescence which has a characteristic yellow-orange emission spectrum [12].

Injection of impermeant probes, such as dextrans, albumin, or fluorescent beads, can be used to gather physiologic information. Figure 1 shows a multiphoton fluorescence image of the kidney of a rat injected with rhodamine-labeled albumin (red), a 10,000-MW fluorescein-labeled dextran (green), and the cell-permeant nuclear probe Hoechst (blue). In this image, the red albumin can be seen retained in the vasculature of the intertubular capillaries and the glomerulus, whereas the small molecular weight green dextran is freely filtered, appearing in both proximal and distal tubular segments. Proximal and distal tubules can be distinguished by several criteria: (1) the filtered dextran is concentrated in the lumen of the distal tubules as compared with the proximal tubules; (2) the filtered dextran is found in punctate endosomes of the endocytically active proximal tubule, and (3) punctate autofluorescent structures are restricted to proximal tubule cells.

Several independent physiological processes are apparent in this single image: (1) glomerular filtration is apparent with the retention of albumin in the vasculature and appropriate filtration of the small dextran; (2) the concentration of solutes in the distal tubule is shown by the increased intensity of the fluorescence of the small dextran; (3) endocytosis in the proximal tubule is reflected in the appearance of the small dextran in punctate endosomes; (4) the intensity of endosome fluorescence is attenuated by their low pH which quenches the fluorescence of fluorescein; (5) the morphology of the labeled nuclei indicates the lack of significant apoptosis which can be recog-

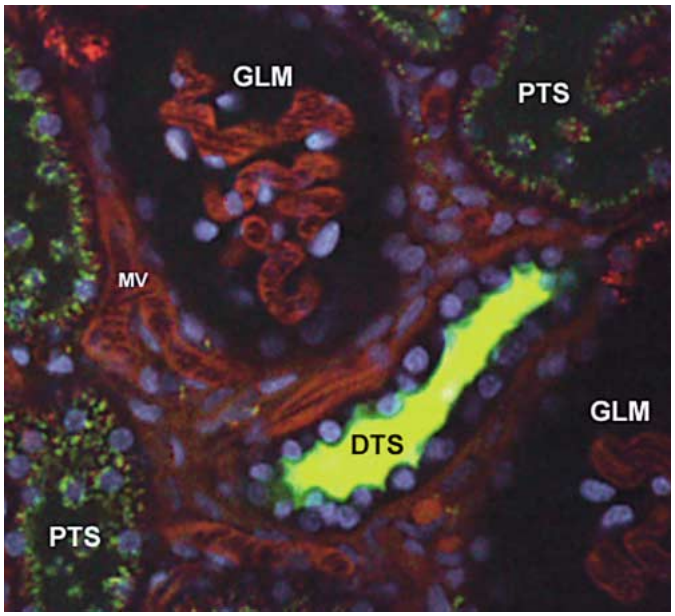

Fig. 1. Intravital two-photon image of a rat renal outer cortex. Cortical section of a rat kidney following intravenous injection with rhodamine-albumin (red), a 10,000-MW FITC-dextran (green), and the nuclear dye Hoechst 33342 (blue), imaged live via two-photon microscopy. GLM = Glomerulus; PTS = proximal tubular segment; MV = microvasculature; DTS = distal tubular segment.

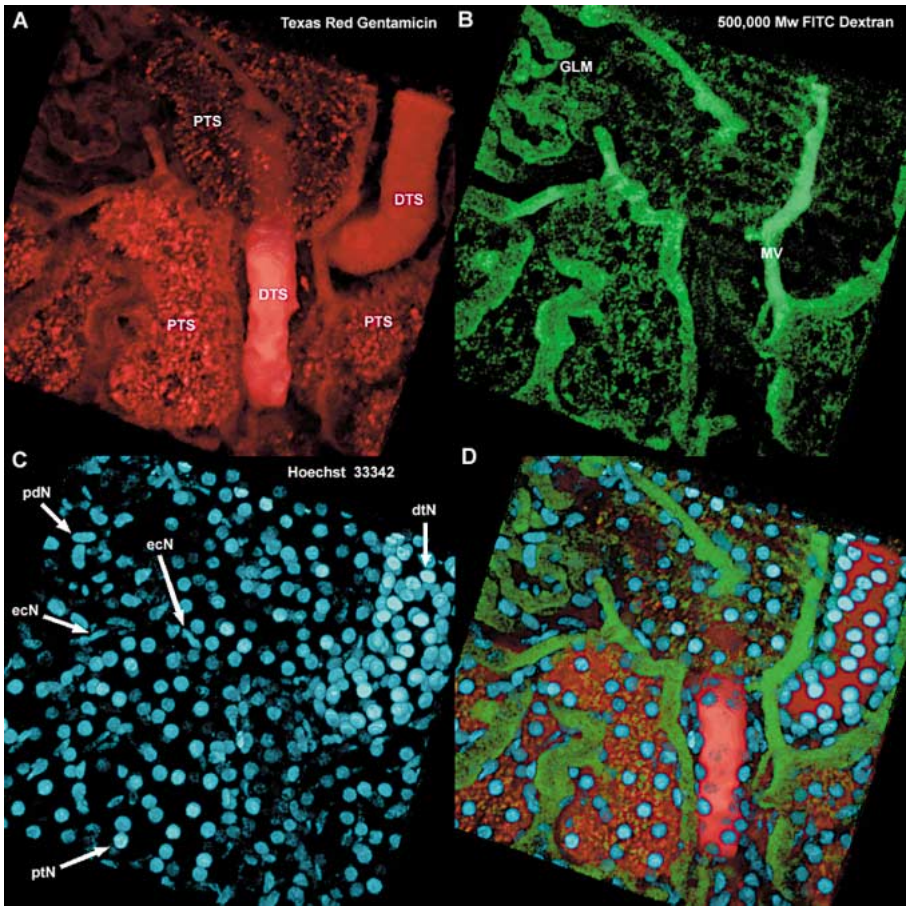

Fig. 2. Three-dimensional reconstruction of intravital two-photon images of a rat renal outer cortex. A four-panel composite of a rat kidney following intravenous injection of Texas Red-gentamicin (red), a 500,000-MW FITC-dextran (green) and Hoechst 33342 (blue), imaged live via two-photon microscopy 4 min later. Optical sections were taken and rendered using VOXX software. Endocytosis of Texas Red-gentamicin (**A**) within the proximal tubular cell produces a punctate staining pattern that differs from the lumenal confinement seen within distal tubular segments. 500,000-MW FITC-dextran (**B**) is retained in the microvasculature. The punctate pattern seen within the PTS is the green component of the autofluorescence seen normally therein. Podocyte nuclei (pdN), endothelial cell nuclei (ecN), and proximal and distal cell nuclei (ptN and dtN, respectively) can be matched with their resident localization within the morphologic structures in the complete overlayed image (**C**, **D**). For explanation of the other abbreviations see legend to figure 1.

nized in the multiphoton images of the kidneys of ischemic rats from their fragmented and irregular nuclei [12], and (6) the pattern of shadows in the intertubular capillaries reflects movement of blood cells.

A critical component of the utility of multiphoton microscopy is its ability to simultaneously utilize multiple fluorescent probes, because of the extensive overlap in the wavelengths of infrared light that can be used to stimulate the fluorescence of different probes. In addition to providing the ability to correlate multiple parameters, the ability to simultaneously image multiple colors of fluorescence is useful for physiological studies, where the use of ratios can correct for extraneous factors in quantitative analyses. Finally, the ability to collect multiple colors of fluorescence is critical to identifying endogenous autofluorescence, the characteristic emission spectrum of which allows it to be identified and distinguished from experimental fluorescent probes [12].

While the most obvious advantage of multiphoton microscopy for intravital imaging is the increased depth at which high-resolution images may be collected from tissues, another advantage is that it may incur less damage to biological tissues. Insofar as fluorescence excitation is limited to a single point in the image volume, photobleaching and attendant oxidative damage are restricted to the plane being imaged, unlike confocal microscopy, in which the photo-oxidation occurs throughout the illuminated field. In addition, the long wavelengths of light used to excite fluorescence in a multiphoton system induce less damage to biological tissues. In particular, Squirrell et al. [11] demonstrated less generation of reactive oxygen in two-photon microscopy as compared with confocal microscopy. The combination of increased depth of penetration and reduced phototoxicity allows for volume collecting and three-dimensional reconstructions, as shown in figure 2.

However, multiphoton microscopy of living animals incurs complications not encountered in studies of isolated tissues or cultured cells. Some of these complications have to do with the practical issues of imaging living animals: (1) the high resolution offered by multiphoton microscopy requires methods to immobilize samples or to avoid sample movement during imaging, and (2) not all tissues of interest are found within the working distance of the microscope objective, typically between 0.2 and 1 mm. Accordingly, in some cases surgical techniques need to be developed to provide access to particular tissues without compromising the tissue function.

Finally, studies of living animals are complicated by the difficulty of delivering probes to specific tissues. While the expression of fluorescent protein chimeras (e.g., green fluorescent protein) avoids this difficulty, correlations are generally desired so that delivery of fluorescent markers is a requirement of nearly any study. In particular, studies of the kidney function will be enhanced by the development of methods to deliver probes to the lumen of specific portions of kidney tubules.

Another family of questions pertain to multiphoton fluorescence excitation itself. Multiphoton microscopy is a new technology, one that is being distributed faster than it has been characterized. The first practical issue in any multiphoton microscopy study concerns the choice of fluorophore and wavelength to be used to stimulate fluorescence for imaging. Multiphoton excitation spectra have been described for relatively few fluorophores [20]. However, the efficiency of fluorescence excitation is only one of many factors that must be considered in selecting the wavelength to excite a given fluorophore. In fact, a quantitative analysis by Patterson and Piston [21] suggests that the optimum wavelength for excitation may need to be chosen as a compromise between excitation and photobleaching efficiencies.

Finally, many aspects of the currently available equipment are not well suited to intravital microscopy. For example, image collection in these single-point scanning systems is relatively slow, confounding their ability to capture dynamics. Slow image capture is a particular problem for collecting three-dimensional image volumes, where the final planes of a volume may be collected minutes after the first planes have been collected. This problem may be addressed with new designs of multiphoton systems capable of collecting images at video rate [22–25]. Issues of scattering, absorption, and refractive index variability that modestly impact imaging of thin samples become preponderant in thick biological tissues, so that unique optical designs may be needed for deep tissue imaging [26].

Concluding Remarks

Developments in optical imaging technologies over the past decade now provide unprecedented opportunities to study the phenotypic expression of cellular components and their interactions. In parallel with this, the development of novel fluorescent probes and methodologies to label specific molecules has allowed the molecular engineering of unique intracellular approaches that can be followed and quantified microscopically. These imaging technologies enable the measurement of the chemical and

biochemical composition of tissues, the expression of functional proteins and enzymes, the rates of biochemical reactions, and the rates of physiological properties such as tissue perfusion or vessel blood flow. Multiphoton microscopy offers the potential to characterize these parameters within the intact organs of living animals.

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